Fine Structural Aspects of the Mobilization of Hepatic Glycogen

II. Inhibition of Glycogen Breakdown

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THE ROLE OF CELLULAR ORGANELLES, especially the endoplasmic reticulum and lysosomes, in glycogen catabolism has been the subject of extensive investigation and dispute.[1-12]

Since massive glycogen breakdown occurs physiologically in the postnatal rat hepatocyte,[13,14] it was hoped that by comparing the ultrastructural changes under normal conditions,[9] conditions of accelerated breakdown,[15] and conditions of inhibited breakdown, it might be possible to delineate which intracellular changes are associated with the breakdown of glycogen in these cells. The present study is concerned with the intracellular changes that occur in postnatal rat hepatocytes when the breakdown of glycogen is inhibited. Glucose and insulin were used to inhibit glycogenolysis. In a preliminary study of the ultrastructural effects of glucose in postnatal rat hepatocytes, delayed formation of lysosomes and inhibition of the normal increase in sparsely-coated vesicles of the endoplasmic reticulum were noted. Some of these findings have been reported in abstract.[16,17]

Since lysosomes and endoplasmic reticulum are the main organelles that have been implicated in the metabolism of glycogen, the activities of acid phosphatase and glucose-6-phosphatase were determined on liver homogenates in these experiments. Biochemical estimations of and glycogen in liver and glucose in blood were also carried out. The electron microscopic findings were quantitated using morphometric analysis.

Materials and Methods

Animals and Handling of Tissues

These were described in detail in the preceding companion paper.[15] In this study, 10 pregnant females were obtained and the average litter contained 10 newborn.

The animals administered glucose were sacrificed at the age of 6 and 12 hours and those administered insulin, at the age of 4 hours. Insulin-treated animals that were not killed died in hypoglycemia at 4½ hours; these animals were not used in this study.

Chemicals

D-Glucose (dextrose) Lot 773372 was obtained from Fisher Scientific Co. Zinc crystalline insulin solution (Insulin-Toronto), 80 units/ml, Lot 1055–3, was obtained from Connaught Medical Research Labs, Toronto, Canada. The reagents for the biochemical determinations and for the electron microscopy were obtained as described in the preceding companion paper.[15]

Biochemical Methods, Electron Microscopy and Morphometric Analysis

A detailed description of these was given in the preceding paper.[15] In addition, morphometric analysis of certain elements of the endoplasmic reticulum as well as of the volume of the cytoplasm per hepatic cell, were performed according to the methods of quantitative stereology.[18–21]

Measurements of the surface area of the limiting membrane of the vesicles of rough endoplasmic reticulum/unit of volume of cytoplasm were made on micrographs enlarged to a final magnification of 41,600. The intersections of the membranes with the horizontal lines of the lattice were counted and the number of intersections/μ of test line overlying the cytoplasm was found. Then the area of membranes included in a unit of volume of cytoplasm was calculated.

The volume of cytoplasm in cu μ/hepatic cell was determined from light micrographs taken from 1-μ-thick sections stained with toluidine blue [22] and enlarged to a final magnification of 1000. First, the volume of hepatic cell cytoplasm/unit of volume of liver tissue was estimated. Second, the number of hepatic cell nuclei/unit of volume of liver tissue was estimated by the method reported by Loud.[20] Then, the ratio of these two estimates was obtained.

This calculation gave the volume of hepatic cell cytoplasm/hepatic cell nucleus. Except for the presence of binucleated cells, this calculation would have given the average cytoplasmic volume/hepatic cell. Since binucleated cells have twice the volume of mononucleated cells, the estimated volume of the hepatic cell cytoplasm/hepatic cell nucleus is still approximately the same as the volume of cytoplasm/hepatic cell.[20] The mean tangent diameter of the hepatic cell nucleus required for the second estimate was found from the average volume-to-surface ratio as described by Weibel.[18] To estimate this ratio, every other segment on the horizontal lines of the lattice was used. (A segment is the part of the line enclosed within two adjacent crosspoints). The intersections of the segments with the nuclear boundaries and the crosspoints of the lattice overlying the nuclear profiles were counted. Then the above ratio was calculated according to the formula of Chalkley as described by Weibel.[18]

Experimental Design, Doses, Routes and Modes of Administration

Two agents, glucose and insulin, were used in this study. The first, glucose, exerts a known inhibitory effect on the postnatal mobilization of glycogen in rat hepatocytes.[13,16] The second, insulin, was found experimentally to retard significantly postnatal mobilization of glycogen under the conditions of our experiments.

The animals were divided into treated and control groups. For each postnatal age studied (4, 6 or 12 hours), approximately equal numbers of experimental animals and controls of the same age and from the same litters were sacrificed during the same experimental period.

The control animals were injected with carrier in Group I (glucose administered) or were untreated normal animals in Group II (insulin administered). The results were statistically evaluated by Student's *t* test.[23] Values of *P* less than 0.05 were considered significant.

Group I: Glucose Administered. A 20% solution of glucose in distilled water was prepared. The newborns were injected intraperitoneally with 0.07 ml of the above solution at 0 hours and every 2 hours thereafter (a dose of 2.33 g/kg each time or 14 mg glucose/injection). Control animals were injected with carrier. All animals were sacrificed at 6 or 12 hours.

Group II: Insulin Administered. Insulin was administered subcutaneously in 0.05 ml of the original solution, which was of a concentration of 80 units/ml. The insulin-treated animals were injected at 0 and 3 hours after birth (a dose of 666.7 units/kg each time) and sacrificed at 4 hours.

Results

Biochemical Results

Administration of glucose resulted in hyperglycemia and the sparing of the liver glycogen, which at 6 and 12 hours remained at high levels. On the contrary, the control animals showed the normal postnatal mobilization of liver glycogen, which by the age of 12 hours led to a virtual depletion of liver glycogen.

Glucose-6-phosphatase activity in the glucose-treated animals did not reach the level of the activity attained by the controls but remained approximately 31% lower. No significant change in the activity of acid phosphatase was noted (Table 1).

Administration of insulin retarded the mobilization of glycogen. At the age of 4 hours, significantly higher levels of glycogen were observed in the liver of treated animals than in that of normal animals of the same age used as controls.

The insulin-treated animals were found to be deeply hypoglycemic compared with the controls. No significant difference in the activities of glucose-6-phosphatase and acid phosphatase between treated and untreated animals was noted at this age (Table 2).

Morphologic Results

Both qualitative and quantitative changes of cellular organelles were studied. The term *lysosomes* includes lysosomes and related particles, from autophagic vacuoles to residual bodies,[24,25] unless otherwise specified. The elements of the rough endoplasmic reticulum described as *ribosome-coated and sparsely coated vesicles* largely include the "mixed" vesicles described by other investigators.[9,26] In the tables of morphometric analysis, the term *glycogen* refers to the hyaloplasmic glycogen unless otherwise specified. In these tables, the various cellular com-

Table 1. Effects, 6 and 12 Hours after Birth, of Administering Glucose to Newborn Rats*

Treatment	Blood glucose (mg/100 ml blood)	Glycogen (mg/mg protein)	G-6-phosphatase (μM P/ mg protein/hr)	Acid phosphatase (μM P/ mg protein/hr)
6 HOURS AFTER BIRTH				
Control	50.8 ± 4.3	0.125 ± 0.035	6.37 ± 1.75	2.74 ± 0.38
	(5)	(5)	(5)	(5)
Glucose	402.2 ± 226.0	0.827 ± 0.140	4.37 ± 1.44	3.19 ± 0.65
	(5)	(6)	(6)	(6)
P	<0.02	<0.001	<0.05	<0.3
12 HOURS AFTER BIRTH				
Control	13.8 ± 6.5	<0.020		
	(3)	(4)		
Glucose	371.0 ± 229.0	0.742 ± 0.183		
	(3)	(3)		
P	<0.1	<0.01		

* Results are means $\pm$ standard deviation. Numbers in parenthesis represent number of observations included in results.

ponents are expressed as percent of cytoplasmic volume. The last column of the tables of morphometric analysis depicts the fraction of the volume of lysosomes and related particles that is occupied by glycogen.

Group I: Glucose Administered

The fine structure of normal rat hepatocytes during the first 12 hours of life has been described by Phillips *et al.*[9] The appearance of the control animals at the ages of 6 and 12 hours differed in no respect from normal animals of the same age.

At 6 hours, the areas of glycogen in the control animals varied in size but generally were reduced compared to those seen at birth. Numerous lysosomes appeared and it is estimated that at least 85% of their volume was occupied by lysosomes of autophagic type. The lysosomes were not distributed randomly throughout the cell but were usually found at the junction of areas of glycogen with the glycogen-free areas and often in the vicinity of the Golgi apparatus. Sometimes they were separated from the hyaloplasmic glycogen by a narrow glycogen-free zone. Noticeable in the glycogen-free areas were ribosome-coated or sparsely coated vesicles. Very few smooth-surfaced vesicles were present and the smooth endoplasmic reticulum as seen in adult hepatocytes was absent in these animals.

At 12 hours, marked changes occurred in the control animals in most

cells. The hyaloplasmic glycogen was totally depleted and the former areas of glycogen completely lost their identity. The number of lysosomes increased notably and they occurred in large clumps. Most of the lysosomes contained only amorphous or membranous electron-dense material and had the appearance of residual bodies.[24] The endoplasmic reticulum was predominantly in the form of ribosome-coated or sparsely coated vesicles. These vesicles appeared much more numerous than those seen earlier. (Fig 1 and 2). The smooth endoplasmic reticulum was absent.

In the glucose-treated animals, the appearance of the hepatocytes was strikingly different from that of the control animals. (Fig 3). At both 6 and 12 hours, enormous hyaloplasmic stores of glycogen were present.

The cytoplasm was divided into large glycogen-containing areas and glycogen-free areas, into which the remaining organelles were crowded. A small number of lysosomes, most of them of the autophagic vacuole type, was noted in contrast with the marked development of this cellular component in the control animals. The autophagic vacuoles were predominantly round and usually filled with glycogen. They usually occurred at the margins of the areas of glycogen and often in the vicinity of the Golgi apparatus. Sometimes, they were separated from the hyaloplasmic glycogen by a narrow glycogen-free zone.

The results of the morphometric analysis are shown in Tables 3 and 4. Large volumes of hyaloplasmic glycogen were observed in glucose-treated animals at 6 and 12 hours. These were comparable to those seen at birth.[15] The volume of lysosomes remained low and the marked post-natal increase, which normally occurs, was not noted. A large fraction of the lysosomal volume was occupied by glycogen, particularly at 12 hours, where 69% of this volume corresponded to engulfed glycogen

Table 2. Effects, 4 Hours after Birth, of Administering Insulin to Newborn Rats*

Treatment	Blood glucose (mg/100 ml blood)	Glycogen (mg/mg of protein)	G-6-phosphatase (μM P/ mg protein/hr)	Acid phosphatase (μM P/ mg protein/hr)
Control	45.8 ± 15.1 (4)	0.223 ± 0.103 (4)	5.03 ± 0.40 (4)	1.57 ± 0.17 (4)
Insulin	<12.5 (4)	0.509 ± 0.072 (4)	4.84 ± 1.17 (4)	1.58 ± 0.26 (4)
P	<0.01	<0.01	>0.5	>0.5

* Results are means $\pm$ standard deviations. Numbers in parentheses represent the number of observations included in results.

(last column of Table 4). In the treated animals, mitochondria and peroxisomes also showed a smaller volume compared with that of controls.

The numerous ribosome-coated and sparsely coated vesicles of the rough endoplasmic reticulum, which were so prominent in the control animals, were not developed to the same degree in the glucose-treated animals.

The surface area of the limiting membrane of the vesicles of rough endoplasmic reticulum/unit of volume of cytoplasm was estimated by morphometric analysis. Three controls· and 3 treated animals were sacrificed at the age of 12 hours. Five blocks from the liver of each animal were used. Two random electron micrographs/block were taken and enlarged as described in the Materials and Methods section. Thus, 30 electron micrographs from the control and an equal number from the treated animals were used (a total number of 2610 counts of intersections for the controls and 2000 counts of intersections for the treated animals). The control animals had 0.87 ± 0.04 sq μ^2 of membrane surface/cu μ of cytoplasmic volume while the treated animals showed only 0.35 ± 0.03 sq μ/cu μ of cytoplasmic volume. The results referred to are the means ± the standard errors. Since, in the glucose-treated animals at the age of 12 hours, the number of all the cytoplasmic organelles decreased (except glycogen, Table 4), the question was raised whether this could be explained on the basis of an absolute increase in the cytoplasmic volume of the hepatocytes of the treated animals. To answer this question, estimations of the cytoplasmic volume/hepatic cell at 12 hours were made by morphometric analysis. For this purpose, five light micrographs from controls (250 hepatic cell

Table 3. Effects, 6 Hours after Birth, on Hepatic Cells of Administering Glucose to Newborn Rats

| Treatment | Percent of cytoplasmic volume* | | | Fraction of glycogen in lysosomes† |
	Glycogen	Lysosomes	Glycogen in autophagic vacuoles	
Control‡	11.3 ± 2.1	1.65 ± 0.20	0.29 ± 0.06	0.18
Glucose§	52.5 ± 3.2	0.42 ± 0.05	0.24 ± 0.07	0.57
P	<0.01	<0.01	>0.5	

* Results are means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 38 micrographs and an area of 6100 sq μ.
§ Results computed from a total of 32 micrographs and an area of 6400 sq μ.

Table 4. Effects, 12 Hours after Birth, on Hepatic Cells of Administering Glucose to Newborn Rats

Treatment	Percent of cytoplasmic volume*					Fraction of glycogen in lysosomes†
	Glycogen	Mitochondria	Peroxisomes	Lysosomes	Glycogen in autophagic vacuoles	
Control‡	<0.07	20.0 ± 0.7	1.03 ± 0.09	5.33 ± 0.80	<0.07	<0.013
Glucose§	39.4 ± 3.0	13.3 ± 0.7	0.78 ± 0.06	0.32 ± 0.15	0.22 ± 0.06	0.69
P	<0.01	<0.01	<0.02	<0.01	<0.01	

* The results are means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 30 micrographs and an area of 5400 sq μ.
§ Results computed from a total of 30 micrographs and an area of 5200 sq μ.

nuclei) and an equal number of light micrographs from treated animals (200 hepatic cell nuclei) were used. The mean volume of cytoplasm/cell in control animals was 4412 cu μ while in glucose-treated animals, it was 5616 cu μ. It is obvious that this increase (approximately 27%) could explain the decrease observed in the relative volumes of the mitochondria and the peroxisomes but not the larger change noted in the lysosomes. The smooth endoplasmic reticulum was absent.

Group II: Insulin Administered

The animals used as controls had the normal appearance expected for the fourth postnatal hour of life.[15] The areas of glycogen were, in general, reduced compared with those seen at birth. The lysosomes occurred usually at the junction of glycogen and glycogen-free areas and often in close relationship with Golgi zones. It was estimated that at least 85% of the lysosomal volume belonged to lysosomes of the autophagic vacuole type.

In insulin-treated animals, the areas of hyaloplasmic glycogen remained vast (Fig 4) and the other cellular organelles were crowded into the glycogen-free areas. Autophagic vacuoles, usually containing relatively large amounts of glycogen, occurred at the margins of the glycogen areas as they did in control animals. They were often in close proximity to the Golgi apparatus.

The results of morphometric analysis are shown in Table 5. The volume of the hyaloplasmic glycogen was increased compared with the normal animals of the same age. No statistically significant difference was found in the volume of lysosomes. In treated animals, the fraction of the volume of lysosomes that was occupied by the engulfed glycogen

Table 5. Effects, 4 Hours after Birth, on Hepatic Cells of Administering Insulin to Newborn Rats

Treatment	Percent of cytoplasmic volume*					Fraction of glyco-gen in lyso-somes†
	Glycogen	Mitochondria	Peroxisomes	Lysosomes	Glycogen in autophagic vacuoles	
Control‡	14.0 ± 2.1	14.5 ± 0.8	0.88 ± 0.06	0.78 ± 0.15	0.13 ± 0.03	0.17
Insulin§	36.4 ± 2.3	17.2 ± 0.9	1.04 ± 0.09	0.56 ± 0.10	0.25 ± 0.05	0.45
P	<0.01	<0.05	<0.1	<0.5	<0.05	

* Results are means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 47 micrographs and an area of 8350 sq μ.
§ Results computed from a total of 41 micrographs and an area of 7200 sq μ.

was increased (last column of Table 5). A small increase in the mitochondrial volume was noted.

Discussion

As was shown from the biochemical results of this study, the normal postnatal mobilization of glycogen in rat hepatocytes was inhibited by the administration of glucose, which is a phenomenon that was known previously.[13,16]

The inhibition of the postnatal mobilization of glycogen by the administration of glucose could be explained on the basis of an abolition of normal postnatal hypoglycemia. The fall in levels of blood glucose after birth has been considered the natural stimulus for the triggering of the secretion of the hormones, glucagon and adrenalin, which activate the enzyme phosphorylase resulting in the breakdown of liver glycogen.[13,27]

In the glucose-treated animals, the activity of glucose-6-phosphatase was lower than that in the control animals. This is in accordance with the findings of Dawkins.[13] This phenomenon could contribute to the inhibition of glycogen breakdown in the postnatal rat hepatocytes. However, glucose-6-phosphatase is not considered to play a key role in regulating this process.[27,28] Inhibiting a rise in this enzyme by using inhibitors of protein synthesis had no effect on the mobilization of glycogen.[13,28]

The inhibition of the postnatal mobilization of glycogen by insulin under the conditions of this experiment can be explained on the basis of the inhibitory action of this hormone on the activity of the key glycogenolytic enzyme, phosphorylase.[29] Insulin given alone, without

simultaneously administered glucose, does not promote synthesis of glycogen.[30] No statistically significant difference in the activity of the enzyme glucose-6-phosphatase was noted in the insulin-treated animals at the age of 4 hours. However, a decreased activity of this enzyme has been reported in animals surviving longer (6 hours after birth) that were treated by a smaller dose of insulin.[13]

The sparing effect of glucose and insulin on the glycogen in newborn liver was reflected in increased volumes of hyaloplasmic glycogen in the hepatocytes of treated animals compared with controls. Lysosomal glycogen similarly increased. Thus, lysosomes were found to be rich in glycogen under conditions of inhibited breakdown of hyaloplasmic glycogen. It was noted again that lysosomes were not distributed randomly throughout the entire cytoplasm, but occurred predominantly at the junction of glycogen areas with glycogen-free areas.[15] Further, it was found, both by morphologic observation and by quantitative morphometry, that administration of glucose results in inhibition of the normal postnatal development of lysosomes. These findings suggest an inhibited catabolism of lysosomal glycogen under these experimental conditions.

In considering the role of lysosomes in breaking down glycogen, it must be remembered that lysosomes (and their derivatives) are known to have many functions.[24,31] For instance, in fetal and perinatal tissues of many types, lysosomal activity has been considered important in differentiation of tissues during development. Therefore, the question must be asked whether the occurrence of glycogen within lysosomes is of any significance in terms of the catabolism of glycogen *per se* or whether its incorporation within the organelles is part of a general cell reconstruction. The evidence in this and the preceding study point to a positive relationship between the catabolism of glycogen in lysosomes and the breakdown of hepatic glycogen in these cells for the following reasons.

Autophagic vacuoles in these cells are not formed randomly but in close relationship to the margins of the areas of glycogen in normal and treated animals. Further evidence is that the number, volume and glycogen content of lysosomes are profoundly influenced both by factors that accelerate [15] and those that inhibit the breakdown of hepatic glycogen. This conclusion is also supported by the observation that depletion of hepatic glycogen (seen in this study in normal animals at 12 hours) is accompanied by a change in most lysosomes from glycogen-containing autophagic vacuoles to residual bodies. This finding suggests completion of the immediate function of these organelles in these cells,

namely, autophagy of glycogen and catabolism of glycogen. The ob-
servations in this study provide further support for the hypothesis
previously made [15] that breakdown of lysosomal glycogen is influenced
by those agents known to regulate breakdown of hyaloplasmic gly-
cogen.

Changes in the activity of acid phosphatase did not occur despite
marked differences in the volume of lysosomes. The lysosomal hydrolytic
enzymes of the autophagic vacuoles are considered to be transferred
from the Golgi apparatus via Golgi vesicles.[25] This view is supported by
the observation that the lysosomes occurred predominantly, in both
control and treated animals, in the vicinity of Golgi zones. According
to the above view, there is no reason *a priori* for the inhibition of the
formation of lysosomes to be associated with a decreased content of
hydrolase of the whole cell. Similarly, the formation of autophagic
vacuoles promoted by glucagon was not associated with an increased
total activity of acid phosphatase in liver homogenates.[15,25]

The inhibition by glucose of the normal increase in the number of
vesicles of rough endoplasmic reticulum may represent a phenomenon
unrelated to the inhibition of the glycogen breakdown, since it was
shown in the preceding companion study [15] that the accelerated break-
down of glycogen was not associated with an increased number of
these vesicles. The inhibition of the normal rise of glucose-6-phosphatase
observed in glucose-treated animals may be related to the inhibition of
the formation of these vesicles of endoplasmic reticulum. However, it
is very unlikely, as discussed before, that these phenomena play a key
role in the regulation of postnatal mobilization of glycogen.

The smooth endoplasmic reticulum, as seen in adult hepatocytes,
was absent in the control animals and no change was observed after
glucose was administered. Hence, this organelle plays no role in
regulating the breakdown of glycogen or release of glucose in these
hepatocytes of postnatal rat. This is in agreement with previous
studies.[9]

The reduced volumes of the mitochondria and peroxisomes ob-
served in glucose-treated animals should not be considered as phe-
nomena associated with the inhibited breakdown of glycogen *per se*.
It is better explained on the basis of an increased absolute volume of
the hepatic cell cytoplasm in these cells. The magnitude of this in-
crease (approximately 27%) is comparable to the decrease in the rela-
tive volumes of the mitochondria and peroxisomes.

A small increase in the volume of mitochondria observed in insulin-
treated animals cannot be explained by the data of this study. Since no

change in the volume of mitochondria was noted after agents modifying the catabolism of glycogen were administered (see also preceding paper [15]), this phenomenon may represent a specific effect of the hormone under the conditions of experiment.

Summary

Relationships between glycogen and organelles in postnatal rat hepatocytes were studied during inhibition of glycogen breakdown induced by glucose or insulin. The observations in this study suggest that an inhibition of the breakdown of hyaloplasmic glycogen is accompanied by inhibition of the breakdown of lysosomal glycogen. When glucose was administered, the inhibition also was associated with an inhibition of the normal postnatal increase in the volume of lysosomes.

These findings constitute good evidence for active participation of lysosomes in the overall breakdown of hepatocellular glycogen in the early postnatal rat. The postulate that those agents that physiologically regulate the breakdown hyaloplasmic glycogen similarly control the breakdown of lysosomal glycogen is supported by these experimental findings.

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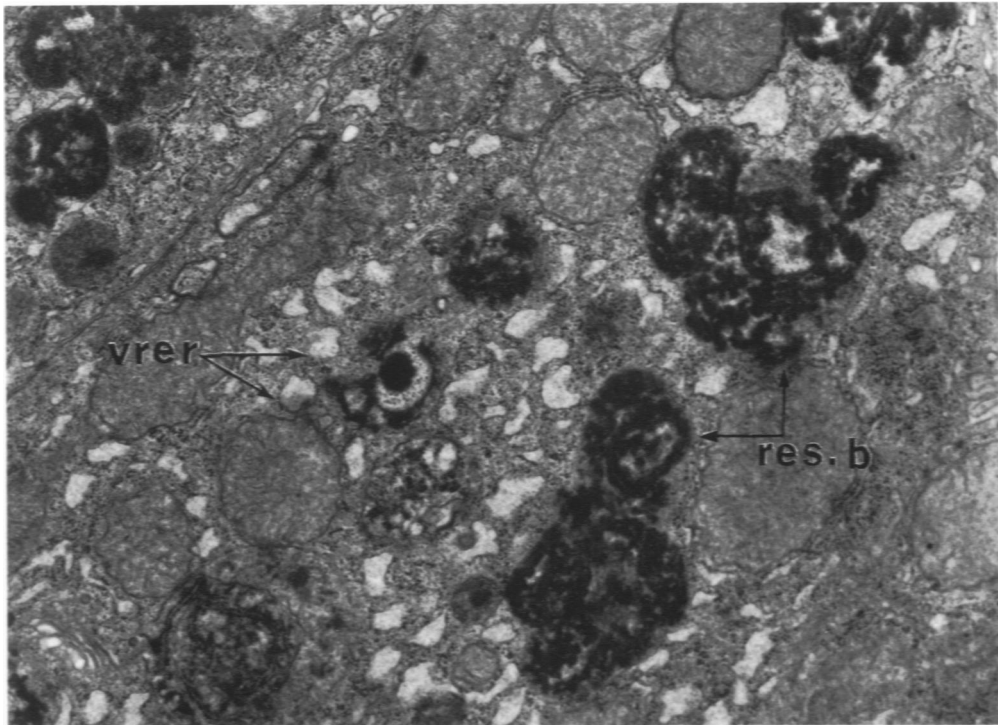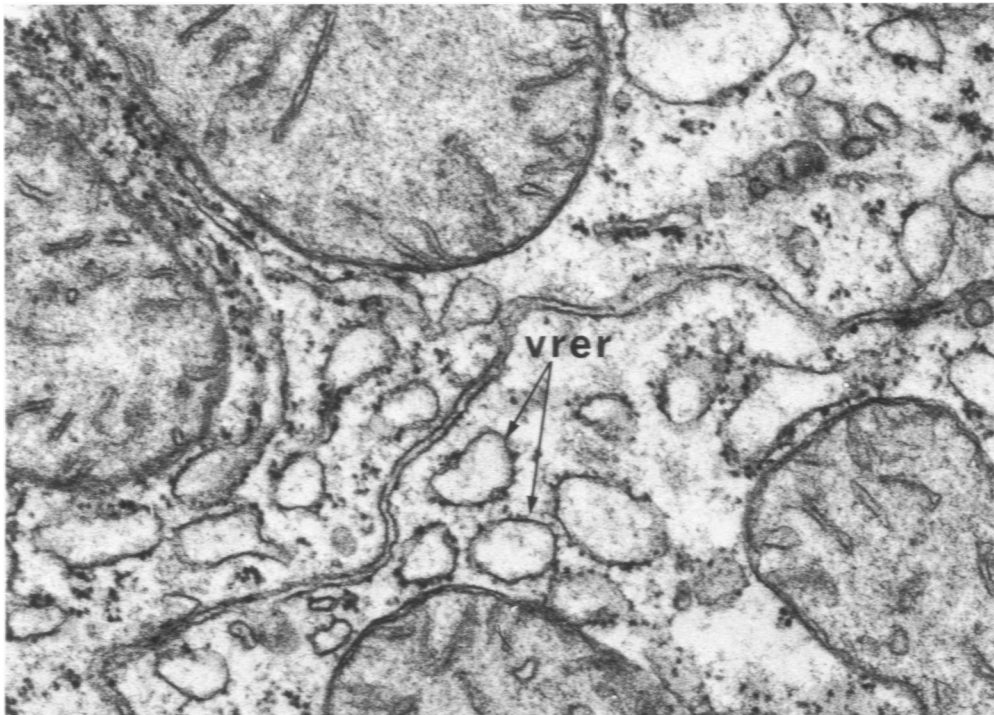
Fig 1.—Portions of two hepatocytes of normal rat at 12 hours. Hyaloplasm is totally depleted of glycogen. Numerous lysosomes, predominantly in the form of residual bodies are present (res. b). Vesicles of rough endoplasmic reticulum (*vrer*) are numerous (uranyl acetate—Reynold's lead citrate, × 13,200).

Fig 2.—Portions of two hepatocytes of normal rat at 12 hours. Many ribosome-coated or sparsely coated vesicles of rough endoplasmic reticulum (*vrer*) are present (uranyl acetate—Reynold's lead citrate, × 41,600).
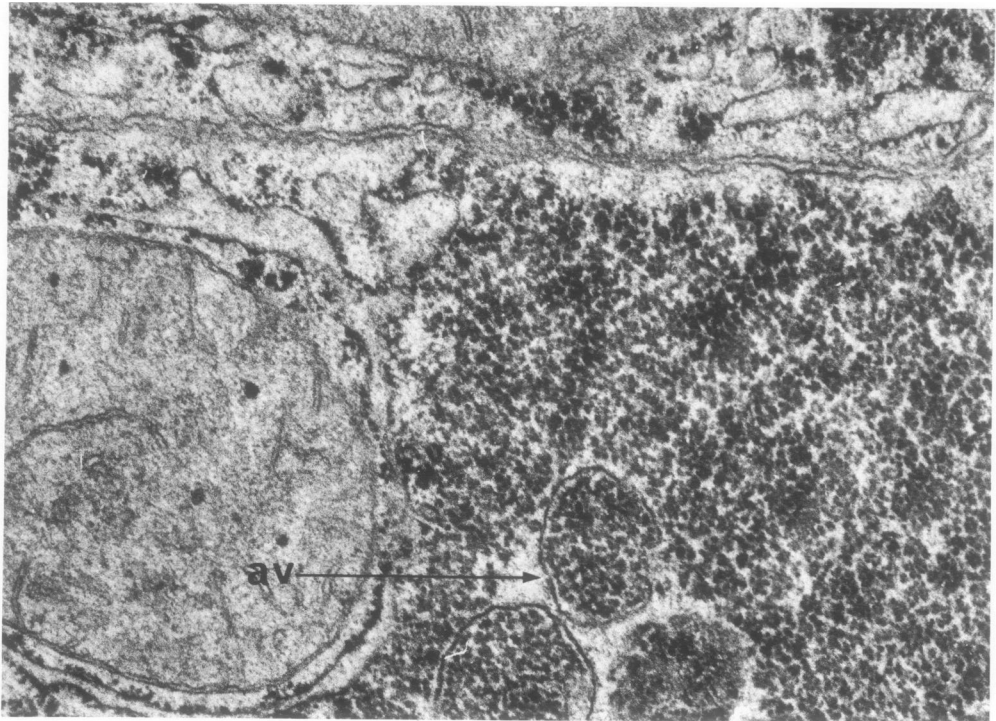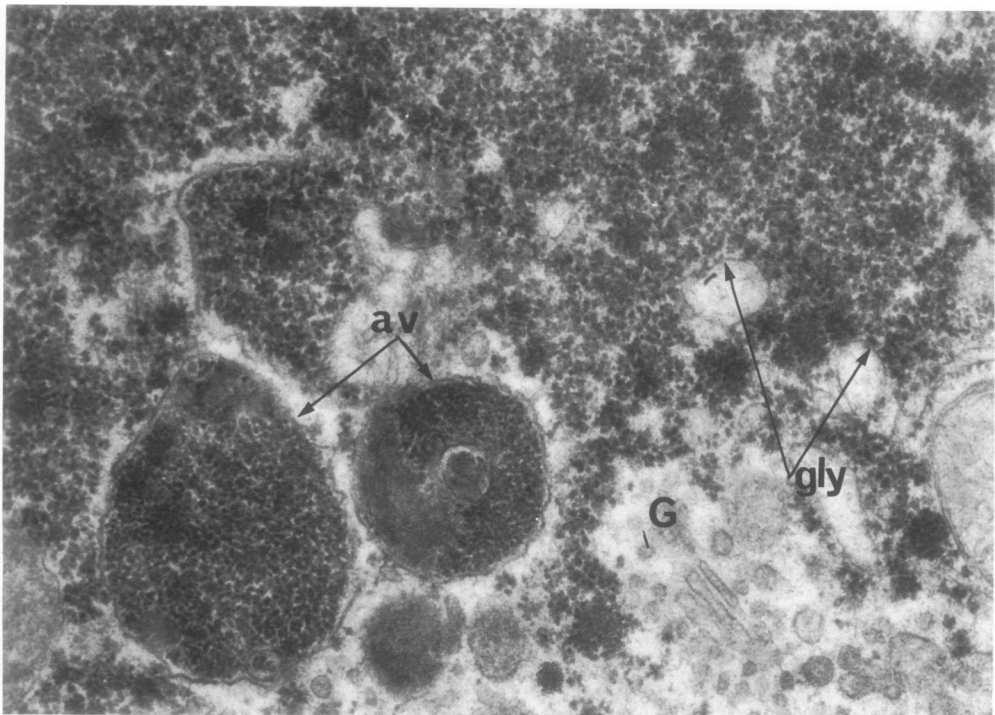
Fig 3.—Portions of two hepatocytes of glucose-treated rat at 12 hours. Autophagic vacuoles (*av*) are small, round and usually filled with glycogen (uranyl acetate—Reynold's lead citrate, × 41,600).

Fig 4.—Portion of hepatocyte of insulin-treated rat at 4 hours. Glycogen (*gly*) is abundant both in hyaloplasm and within autophagic vacuoles (*av*). Note that autophagic vacuoles are near the margin of the glycogen area. Golgi zone (*G*) is seen in vicinity of vacuoles (uranyl acetate—Reynold's lead citrate, × 24,000).